Remarks

Claims 1-11 and 19-22 were pending. Claims 1, 7 and 19 have been amended to correct minor informalities, and clarify the intended subject matter in accordance with the Examiner's suggestions. No new matter is added. Applicants respectfully request reconsideration of the rejections.

Claims 1, 7 and 19 have been objected to. The informalities noted by the Examiner have been corrected. Withdrawal of the objection is requested.

Claims 1-11 and 19-22 have been rejected under 35 U.S.C. 112, first paragraph. The Office Action states that the specification is enabling for a bipotent mammalian common lymphoid progenitor cell, but not for a multipotent cell capable of giving rise to T cells, B cells and NK cells. Applicants respectfully submit that the present application meets the requirements of 35 U.S.C. 112, in providing one of skill in the art with a written description of a common lymphoid progenitor that is capable of giving rise to B, T and NK cells.

As shown in Figure 2B of the specification, the cells of the present invention give rise to B cells, T cells and NK cells (see also page 4, line 24-25 and page 18, line 23). These data clearly demonstrate that NK cells arise from the CLP cell population, in addition to B cells and T cells.

With respect to single cell assays, it has been demonstrated by the inventors (published in Kondo *et al.* (1997) Cell 91:661-672) that aliquots from a colony derived from a single CLP cell, when injected into the thymus, could give rise to T cells. The B cells remaining in the colony formed B cell colonies.

It is further demonstrated in a single cell assay by the inventors (published in Kondo *et al.* (2000) Nature 407:383-386; supplemental figure entitled "single cell assay", copy enclosed) that a substantial number of wild-type CLP give rise to mixed colonies of B and NK cells. Therefore, a single cell is capable of giving rise to B cells and NK cells; and single B cell colonies can give rise to T cells, when provided with an appropriate microenvironment.

The Office Action cites Maki *et al.* (1996), which reference teaches that animals deficient in IL-7 receptor have $\alpha\beta$ T cells and have B cells, but at reduced levels along with normal levels of NK cells. It is suggested by the Examiner that these data could be extrapolated to "cast doubt as to whether the very progenitor that gives rise to T- and B-cells would also give rise to NK cells", (although Applicants note that such an inference is not put forward by the authors of the paper).

Applicants respectfully reject this logic. The fact that a particular cytokine influences the ultimate development of a particular differentiated cell is not relevant to the character of progenitor cells or stem cells that give rise to the differentiated cell. Would the teachings of Maki *et al.* also suggest that NK cells cannot arise from a hematopoietic stem cell, since such a stem cell also gives rise to B cells and T cells? Surely the same logic could apply, and yet would fly in the face of well-established experimental results demonstrating that these cells arise from a common stem cell.

The data from the present application makes it very clear that a progenitor cell, having the phenotype set forth in Claim 1, and which normally expresses the IL-7Rα, gives rise to NK cells. Whether these cells can successfully differentiate in the absence of IL-7 receptor signaling is not relevant to the identity of the progenitor cell, and is not relevant to the identity of other cells that differentiate from the same common lymphoid progenitor.

Applicants respectfully submit that the subject application meets the requirements of 35 U.S.C. 112 for a claim to a composition, wherein an individual progenitor cell in said composition is capable of giving rise to each of T cells, B cells, and natural killer cells. Withdrawal of the rejection is requested.

The Office Action states that "it is necessary for applicants to provide support for claimed markers recited in claim 5 and 22, because the specification only prophetically teach that these markers could be used for identifying the common lymphoid progenitor cells". Applicants respectfully submit that such a showing of support is not necessary under 35 U.S.C. 112. The markers set forth in Claims 5 and 22 are characteristic of the cells identified by the methods of the invention. There is no reason for one of skill to doubt that these markers are present on the common lymphoid progenitor cells.

Indeed, it is known in the art that CD45 is expressed on all non-erythrocyte cells of hematopoietic origin, including hematopoietic stem cells. Further, Kondo *et al.* (1997), *supra.* have published (in Figure 2) experimental data relating to the low levels of expression for HSA and CD43 in common lymphoid progenitor cells.

Applicants respectfully submit that the subject application meets the requirements of 35 U.S.C. 112 for claims 5 and 22. Withdrawal of the rejection is requested.

Claims 1-11 have been rejected under 35 U.S.C. 112, second paragraph. The Office Action states that the recited individual progenitor cell is not limited by the surface markers set forth in the same claim. Independent Claims 1 and 7 have been amended to clarify the

phenotype of the independent progenitor cell. In view of the above amendments and remarks, withdrawal of the rejection is requested.

Claims 1-3, 6-10 and 19-20 have been rejected as unpatentable over Olweus *et al.* (US 6,555,324) in view of Galy et al. (US 5,972,627). The Office Action asserts that Olweus *et al.* teach that "progenitors committed to lymphoid lineage" can be identified with markers of CD34 and CD38, plus IL-7 receptor; and that Galy teach that such cells are $CD34^+$, $CD38^+$, $CD45^+$, Lin^-, $Thy\text{-}1^-$, $c\text{-}kit^{low}$. The Office Action concludes that it would have been obvious to one of skill in the art at the time the invention was made to combine the methods taught by Olweus *et al.* and Galy *et al.* Applicants respectfully submit that the presently claimed invention is not made obvious by the cited combination of references.

In response to Applicants' previously filed response, the Office Action states that "one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references". Applicants respectfully submit that, just as one cannot *solely* consider references separately; one cannot also *solely* consider references in combination. The nature of the art, the unpredictability of the field, the motivation to combine references, and specific teachings of the references must all be taken into account.

The Supreme Court has frequently warned against the use of "hindsight" in determining obviousness (*Skega Aktiebolag v. B. F. Goodrich Co.* 420 F.2d 1358, 164 USPQ 333 (6th Cir. 1970), cert. denied, 400 U.S. 825 (1970)). Decomposing an invention into its constituent elements, finding each element in the prior art, and then claiming that it is easy to reassemble these elements into the invention, is a forbidden ex post analysis. Unless the prior art itself suggests the particular combination, it does not show that the actual invention was obvious or anticipated.

The field of stem and progenitor cells is unpredictable. In dealing with a biological system, such as the lineages involved in hematopoiesis, it is difficult to predict what markers might be present on a cell. One must ask, was there a reasonable expectation of success, based on the teachings of the art, that a single common lymphoid progenitor cell existed, and that if it did, would IL-7 receptor be expressed on the cell surface? Further, with respect to the method claims, was there a reasonable expectation that the cited markers would be useful in the isolation of a common lymphoid progenitor? Applicants respectfully submit that the teachings of the art do not provide the requisite reasonable expectation of success.

With respect to the single common lymphocyte progenitor cell, one of ordinary skill in the art would not have been provided with a reasonable expectation that the cell populations

described in either of Olweus *et al.* or Galy *et al.* contained individual cells which could give rise to any of B-, T- or NK-cells. The Office Action quotes Galy et al. "we *speculate* that a common clone . . . *might* be found." (emphasis added) Such speculations and possibilities do not provide a reasonable expectation that such a cell exists.

The present claims require that an individual cell in the claimed population have the capability of giving rise to any of the cited lymphoid cells, and therefore it is incorrect to state, as the Office Action does, that "as long as the combined teachings reasonably taught identifying a lymphoid progenitor cell with the recited markers, they met claim limitation, *i.e.* the cell population identified would comprise the multipotent common progenitors."

As stated by Applicants in the previous response, Olweus *et al.* fails to provide <u>any</u> evidence that its cell population of "progenitor cells committed to the lymphoid lineage" can, in fact, give rise to B-, T- and NK-cells, much less distinguishing between the possibilities of a mixed population of unipotent lymphoid progenitors and a population of individual multipotent lymphoid progenitors. There is not a single experimental example in Olweus *et al.* that demonstrates its "progenitor cells committed to the lymphoid lineage" can actually give rise to any of B-, T- or NK-cells, much less that any single cell in that population could give rise to all three lymphoid cell types.

The Office Action suggests one of skill in the art would combine Olweus *et al.* with Galy *et al.* Apparently, the only experiments performed by Olweus *et al.* were staining and flow cytometry of a fetal bone marrow population, in the absence of any characterization of the developmental potential of the population. One of skill in the art can conclude nothing from these stainings, other than that, perhaps, $CD38^+$ cells may be IL-7R negative or positive (shown in Figure 6A). This is hardly sufficient to teach the phenotype and developmental potential of the presently claimed common lymphoid progenitor cells.

The Examiner would have one believe that the importance of IL-7 in lymphoid lineage development would make it obvious to combine the teachings of Olweus *et al.* with the markers of Galy *et al.* Applicants respectfully submit that, in fact, the role of IL-7 in lymphoid lineage underscores the unpredictability of whether a particular marker will be absent or present on a progenitor cell. As shown by the reference provided by the Examiner, Maki *et al.* (1996) PNAS 93:7172-7177, mice deficient in IL-7 receptor (a) have reduced but detectable numbers of $\alpha\beta$ T cells and B cells and (b) have normal levels of NK cells. One may conclude from this that IL-7 receptor is involved but not critical to differentiation of B cells and T cells, and that IL-7 receptor is not involved in the differentiation of NK cells. It is therefore quite unexpected to find, as Applicants did, that the IL-7Rα is a marker for a progenitor cell common to these lineages.

In view of the above amendments and remarks, Applicants respectfully submit that the presently claimed invention is not made obvious by the cited combination of art. Withdrawal of the rejection is requested.

Applicants submit that all of the claims are now in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, she is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number STAN-064.

Respectfully submitted,

Date: Sept. 9, 2004

By: Pamela Sherwood

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EXHIBIT 1

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Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow

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Summary

The existence of a common lymphoid progenitor that can only give rise to T cells, B cells, and natural killer (NK) cells remains controversial and constitutes an important gap in the hematopoietic lineage maps. Here, we report that the Lin$^-$IL-7R$^+$Thy-1$^-$Sca-1^{lo}c-Kit^{lo} population from adult mouse bone marrow possessed a rapid lymphoid-restricted (T, B, and NK) reconstitution capacity in vivo but completely lacked myeloid differentiation potential either in vivo or in vitro. A single Lin$^-$IL-7R$^+$Thy-1$^-$Sca-1^{lo}c-Kit^{lo} cell could generate at least both T and B cells. These data provide direct evidence for the existence of common lymphoid progenitors in sites of early hematopoiesis.

Introduction

Adult bone marrow contains hematopoietic stem cells (HSCs), which can give rise to all lymphocyte populations, as well as other blood cell types (reviewed by Thomas, 1991). In mice, it has been possible to identify and isolate a purified population of HSCs (for reviews, see Visser and Van Bekkum, 1990; Morrison et al., 1995) The Thy-1.1^{lo}lineage(Lin)$^{-/lo}$Sca-1$^+$ population represents only ~0.05% of mouse bone marrow cells, yet upon transplantation, these cells can fully reconstitute all blood cell elements and radioprotect lethally irradiated congenic hosts (Spangrude et al., 1988). In C57BL/Ka-Thy1.1 mice, this population contains all of the clonogenic multipotent progenitors (Uchida and Weissman, 1992). A single HSC can repopulate all blood cell types (Smith et al., 1991; Osawa et al., 1996). Mouse HSCs have been further subdivided into three populations: long-term HSCs constitute ~0.005%–0.01% of mouse bone marrow (Harrison and Zhong, 1992; Morrison and Weissman, 1994; Osawa et al., 1996) and can self-renew virtually for life, short-term HSC populations have a limited self-renewal capacity, while multipotent (lymphomyeloid) progenitors do not self-renew (Morrison and Weissman, 1994; Morrison et al., 1997). The three populations of HSCs can be arranged in a lineage, according to a progressive loss of ability to self-renew. The expression markers that are used for purifying these subpopulations within the HSCs include c-Kit (Ikuta et al., 1991; Okada et al., 1991; Ikuta and Weissman, 1992), CD34 (Osawa et al., 1996), incorporation of mitochondrial supravital dye Rhodamine-123 (Mulder and Visser, 1987; Spangrude and Scollay, 1990; Li and Johnson, 1992), and/or lack or low-level expression of the lineage-associated antigens such as CD4 and Mac-1 (Morrison and Weissman, 1994).

In addition to the loss of self-renewal activity, the coordinated changes of developmental potential of HSCs might also include the irreversible restriction of one or more lineage commitments owing to sequential activation or silencing of various genes (reviewed by Clevers and Grosschedl, 1996; Shivdasani and Orkin, 1996), including genes related to expression of various cytokine receptors (McKinstry, et al., 1997). The identification of oligopotent hematolymphoid progenitors has been difficult, since the evaluation of differentiation potential may be complicated by a failure to detect a differentiation into particular lineages owing to the limitation of the assays available. These bipotent or oligopotent hematolymphoid progenitors may not reach suitable microenvironments in vivo, or may undergo insufficient expansion for detection in vivo, or appear to contain variable differentiation potentials owing to the stochastic nature of lineage commitment, at least in vitro (Nakahata et al., 1982; Suda et al., 1984). Accordingly, the evidence for bipotent or oligopotent progenitors should include demonstration of a strict bi- or oligopotent functional ability that cosegregates with a distinct cell population definable by some markers such as surface phenotypes, and clonal studies showing bi- or oligolineage outcomes from single cells contained in that population.

It is still unclear whether T and B cell development could or must derive from clonogenic lymphoid-restricted stem cells or from common lymphoid progenitors (CLPs), or if they derive from multipotent progenitors. The CD10$^+$CD34$^+$Lin$^-$c-Kit$^-$Thy-1$^-$ population in human bone marrow gives rise to T, B, NK, and lymphoid dendritic (LD) cells, but very few (if any) myeloerythroid cells; they include clonal progenitors for B, NK, and LD cells (Galy et al., 1995), but it is unknown if the single clonogenic cell of this phenotype gives rise to T cells. In the murine thymus, as a population, the most immature Thy-1^{lo}CD4^{lo}CD25$^-$CD44$^+$ or Thy-1^{lo}c-Kit$^+$ thymic precursors can generate all T, B, NK, and LD cells (Wu et al., 1991a; Ardavin et al., 1993; Matsuzaki et al., 1993), and the NK1.1$^+$c-Kit$^+$CD25$^-$CD44$^+$ cells in fetal thymus can generate both T and NK cells (Carlyle et al., 1997). However, clonal analysis of the outcomes of differentiation from these cells have not been reported. Humans with an adenosine deaminase deficiency (Fischer, 1992; Markert, 1994) or mice with ablations or modifications of specific genes such as *Ikaros* and the common cytokine receptor γ chain (γ_c) lack all three lymphoid classes (Georgopoulos et al., 1994; Cao et al., 1995; DiSanto et al., 1995; Ohbo et al., 1996), but absence of different cells by single gene mutations do not prove existence of a common progenitor (Weissman, 1994). Thus, to date no direct evidence of the existence of cells with a capacity to generate both T and B lymphocytes, but not myeloerythroid cells, has been reported (Shortman and Wu, 1996). We wished to test whether CLPs may exist in sites of early lymphopoiesis at a clonal level.

*To whom correspondence should be addressed.

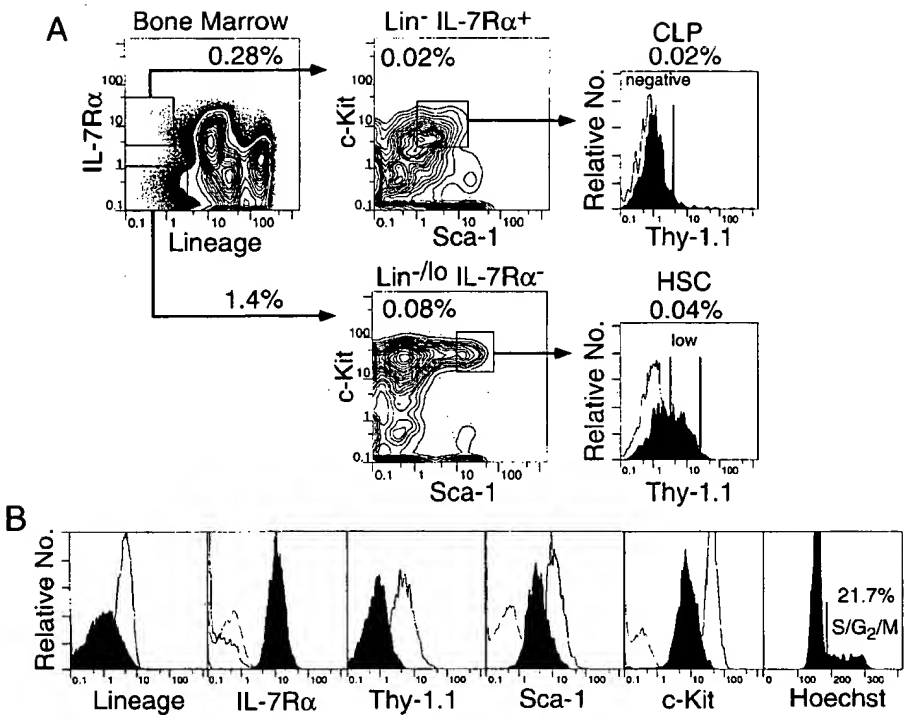

Figure 1. Identification of IL-7Rα$^+$ Common Lymphoid Progenitors (CLPs) in Mouse Bone Marrow

(A) The cells that stained in the negative to low range for lineage markers (B220, CD4, CD8, CD3, Gr-1, Mac-1, and TER119) were subdivided into IL-7Rα positive and negative fractions (left). Sca-1 and c-Kit profiles of each fraction showed the presence of Lin$^-$IL-7R$^+$Sca-1^{lo}c-Kit^{lo} cells (top center panel) and Lin$^{-/lo}$IL-7Rα$^-$Sca-1^{hi}c-Kit^{hi} cells (bottom left panel). More than 90% of the Lin$^-$IL-7R$^+$Sca-1^{lo}c-Kit^{lo} cells were Thy-1.1$^-$ (top right panel), and the CLP activity was found in the Thy-1.1$^-$ fraction (data not shown). HSCs were purified from the Thy-1.1^{lo} fraction of Lin$^{-/lo}$IL-7Rα$^-$Sca-1^{hi}c-Kit^{hi} cells (bottom right panel) (Morrison and Weissman, 1994).

(B) The surface phenotype on reanalysis of the sorted candidate CLPs (closed histogram) and HSCs (solid line) and the cell cycle status of the CLP population by Hoechst 33342 staining; dashed lines show negative staining of whole bone marrow cells. The candidate CLPs were Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo}, and ~22% of them were in S/G2/M phase of cell cycle.

Interleukin 7 acts as a nonredundant cytokine for both T cell and B cell development (Peschon et al., 1994; von Freeden-Jeffry et al., 1995) by maintaining cell survival for T cell precursors (Akashi et al., 1997; Kondo et al., 1997; Maraskovsky et al., 1997; von Freeden-Jeffry et al., 1997) as well as by supporting proliferation (Namen et al., 1988; Sudo et al., 1989) and rearrangement of immunoglobulin heavy chain (*IgH*) genes for B cell precursors (Corcoran et al., 1996). IL-7 exerts its effect through the interaction of a high affinity receptor complex composed of the IL-7 receptor (IL-7R) α chain (IL-7Rα) and the common cytokine receptor γ chain (Noguchi et al., 1993; Kondo et al., 1994). Because of the pivotal role of the IL-7R-mediated signals in lymphoid development, we searched for CLPs in C57BL/Ka-Thy1.1 mouse bone marrow using IL-7Rα expression as a marker. We demonstrated a newly identified Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} population that possessed rapid and prominent short-term lymphoid-restricted (T, B, and NK cells) reconstitution activity. A clonogenic analysis of the cells revealed that single Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells could differentiate into both T and B cells. Thus, this population represents the earliest known lymphoid-committed progenitor in mouse bone marrow.

Results

Identification of the Lin$^-$IL-7R$^+$Thy-1.1$^-$ Sca-1^{lo}c-Kit^{lo} Population

We sorted the bone marrow cells in C57BL/Ka-Thy1.1 mice, using IL-7Rα expression as a marker in addition to conventional HSC markers including Lin, Thy1.1, Sca-1, and c-Kit (Morrison and Weissman, 1994). Figure 1A shows expression profiles of bone marrow cells for these markers. The majority of cells expressing IL-7Rα were B220$^+$CD43$^+$IgM$^-$ pro-B and B220$^+$CD43$^-$IgM$^-$ pre-B cells (Sudo et al., 1993; data not shown). Among the population of cells that did not express lineage markers (Lin$^-$), the IL-7Rα$^+$ fraction contained a population of Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells that made up ~0.02% of total bone marrow cells. All of these IL-7Rα$^+$ cells expressed the γ_c (data not shown), indicating that they expressed functional IL-7R complexes. The Lin$^{-/lo}$IL-7Rα$^-$ fraction contained Thy-1.1^{lo}Sca-1^{hi}c-Kit^{hi} cells, which included HSC subsets (Morrison and Weissman, 1994) (Figure 1A). Figure 1B shows a result of reanalysis of the sorted Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} population and the Lin$^{-/lo}$IL-7Rα$^-$Thy-1.1^{lo}Sca-1^{hi}c-Kit^{hi} population.

The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells had rearranged neither immunoglobulin heavy chain (*IgH*) nor

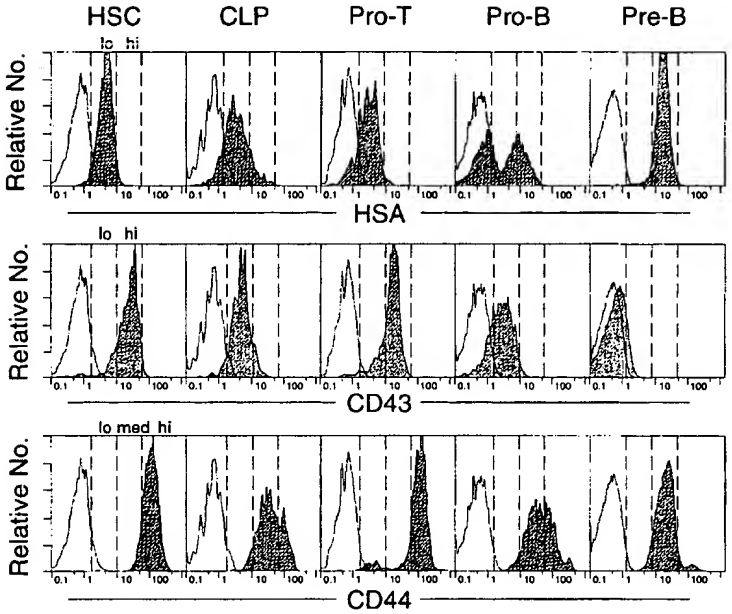

Figure 2. The Expression of HSA, CD43, and CD44 in HSCs, CLPs, and Various Lymphoid Progenitors

The definition of thymic pro-T population used here is CD3-CD4$^{-/lo}$CD8$^{-/lo}$CD25^{-}c-Kit^{+}, and that of either bone marrow pro-B or pre-B population is B220^{+}IgM^{-}CD43^{+} and B220^{+}IgM^{-}CD43^{-}, respectively (see Experimental Procedures). The gray filled histograms represent the expression profiles of these antigens in each population. Solid lines indicate negative stainings. Since most Lin^{-}IL-7R^{+}Sca-1^{lo}c-Kit^{lo} cells were Thy-1.1^{-} (Figure 1), the stainings of HSA, CD43, and CD44 for CLPs were done for Lin^{-}IL-7R^{+}Sca-1^{lo}c-Kit^{lo} cells.

T cell receptor β (*TCR*β) genes determined by PCR analysis (data not shown), and ~22% of the cells were in S/G2/M phase of cell cycle, indicating that considerable fractions are cycling (Figure 1B). Figure 2 shows the expression of heat-stable antigen (HSA; CD24), CD43, and CD44, all have been used previously for identifying either thymic pro-T cells or pro-B cells in bone marrow (Hardy et al., 1991; Wu et al., 1991b). The majority of the Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} cells expressed a low level of CD43 and a medium level of CD44. In contrast, both HSCs and the CD3^{-}c-Kit^{+}CD25^{-} earliest thymic pro-T precursors (Wu et al., 1991b; Godfrey et al., 1992; Matsuzaki et al., 1993) expressed higher levels of CD43 and CD44. The B220^{+}IgM^{-}CD43^{+} pro-B cell population in the bone marrow could be divided into HSA^{-} and HSA^{hi} populations, the former of which corresponded to the most primitive pro-B stage (pre-pro-B; fraction A), according to the classification proposed by Hardy et al (1991). The Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} cells as well as HSCs and the thymic precursors expressed low levels of HSA. Accordingly, the Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} cells are distinguishable from pro-T and pro-B populations as well as HSCs in terms of expression profiles of these surface markers.

The Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} Cells Possess Rapid and Prominent Lymphoid-Restricted Reconstitution Potential

To evaluate the differentiation capacity of the the Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} cells, we competitively reconstituted lethally irradiated (920 rad) Ly5.1 mice with 2 × 10^{3} Ly5.2 congenic Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} cells and 2 × 10^{5} Ly5.1 syngeneic bone marrow cells. Figure 3 shows the serial changes of mean percentages of chimerism in blood nucleated cells after the competitive reconstitution. All through the analysis, we detected exclusively donor-derived T and B cells, but donor-derived Gr-1^{+} granulocytes or Mac-1^{+} monocytes never appeared in the blood. The peak level of donor-type (Ly5.2^{+}) B cells was apparent at 4 weeks (the earliest point of analysis) and of T cells at 4–6 weeks after injection. The percentage of both donor-derived T and B cells gradually decreased, indicating that their self-renewal potential is limited. There was no particular Vβ bias in the donor-derived T cells; they consisted of percentages of Vβ3-, Vβ6-, Vβ8-, and Vβ11-positive cells similar to normal mice (data not shown).

In the competitive reconstitution assay, the coinjected host-type bone marrow cells contained mature lymphocytes as well as a variety of stages of bone marrow

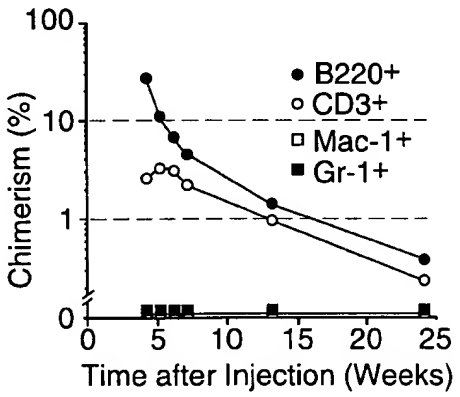

Figure 3. Reconstitution Potential of the Lin^{-}IL-7R^{+}Thy-1.1^{-}Sca-1^{lo}c-Kit^{lo} CLP Population to Lethally Irradiated Congenic Mice

Sequential analysis of blood cell chimerism after competitive reconstitution with 2 × 10^{3} CLPs (Ly5.2) and 2 × 10^{5} syngeneic (Ly5.1) bone marrow cells. Mean percentages of donor-derived cells positive for B220 (closed circles), CD3 (open circles), Mac-1(open squares), and Gr-1 (closed squares) are shown (n = 4). The B220^{+} cells in blood were surface IgM^{+} in all sample tested. Neither Mac-1 nor Gr-1 positive cells were detectable.

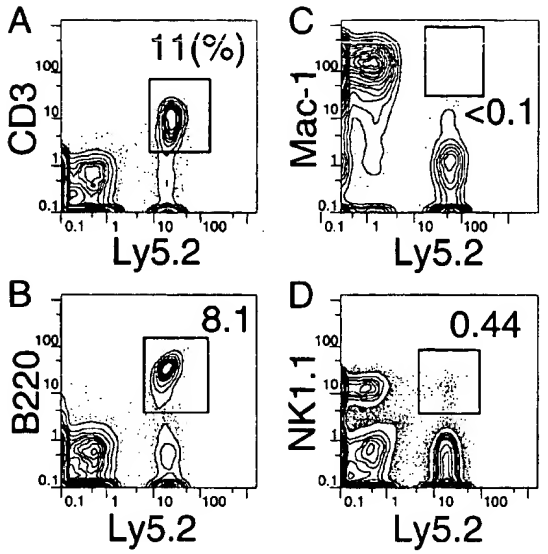

Figure 4. Reconstitution Potential of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP Population (Ly5.2) to Sublethally Irradiated *RAG*$^{-/-}$ (Ly5.1) Mice

(A–C) Phenotypic analysis of nucleated blood cells 4 weeks after intravenous injection of 400 candidate CLPs (Ly5.2) into sublethally irradiated (400 rad) *RAG*$^{-/-}$ (Ly5.1) mice are shown. The number of donor-type (Ly5.2) CD3$^+$ (A) and B220$^+$ (B) cells reached 176 cells/μl and 130 cells/μl of blood, respectively, but Mac-1$^+$ cells (C) were absent in the blood

(D) CD3$^-$NK1.1$^+$ cells were also detected in the spleen of the same mouse 8 weeks after injection (the profile of CD3$^-$ spleen cells is shown).

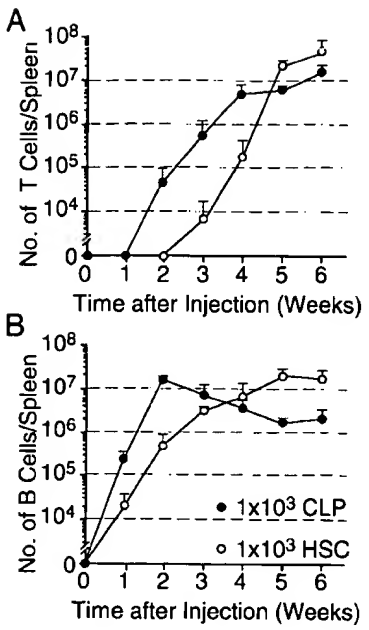

Figure 5. The Sequential Analysis of Numbers of T Cell and B Cell Progeny from CLP and HSC Populations

Absolute numbers of the donor-derived CD3$^+$ T cells (A) or B220$^+$ B cells (B) in the spleen of sublethally irradiated *RAG-2*$^{-/-}$ mice injected with either 10^3 Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLPs (closed circles) or 10^3 Lin$^{-/lo}$IL-7R$-$Sca-1^{hi}c-Kit^{hi} HSCs (open circles) were shown. The highest number of CD3$^+$ progeny that was generated from 10^3 CLPs was $\sim 1 \times 10^7$ at 4–6 weeks, while that of B220$^+$ progeny was $\sim 1.5 \times 10^7$ at 2 weeks. Results are shown as mean plus S.D. (error bars) from groups of 3–5 mice at each time point.

precursors including HSCs. We considered the possibility that the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells failed to reach suitable bone marrow microenvironments to read out myeloid outcomes because the stromal niche may be immediately occupied by host-type progenitors or that myeloid differentiation from this population may be perturbed by inflammatory cytokines released from activated lymphocytes as well as damaged stromal cells owing to lethal-dose irradiation (Antin and Ferrara, 1992). To exclude these possibilities, we reconstituted sublethally irradiated syngeneic *RAG-2*-deficient (*RAG*$^{-/-}$) mice that lack T and B cells only with the candidate CLP population. We first injected 400 Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells (Ly 5.2) to sublethally irradiated (400 rad) Ly 5.1 *RAG*$^{-/-}$ mice. Four weeks after injection, the mice exhibited donor-type reconstitution only for T and B cells, but not myeloid cells (Figure 4). Interestingly, as few as 400 cells could reconstitute the number of blood T and B cells to 5%–20 % of levels in normal mice. We could also detect donor-derived CD3$^-$NK1.1$^+$ NK cells in the spleen in all 4 mice analyzed 8 weeks after transplantation (Figure 4), indicating that the CLP population can give rise to NK cells as well as to T and B cells.

Next, we evaluated the changes in absolute numbers of T and B progeny in the spleen in the *RAG*$^{-/-}$ mice that were reconstituted with either 10^3 CLP or 10^3 HSC (Lin$^{-/lo}$IL-7R-Thy-1.1^{lo}Sca-1^{hi}c-Kit^{hi}) populations. As shown in Figure 5, 10^3 CLPs could generate $0.6\sim1.1 \times 10^7$ CD3$^+$ spleen T cells by 4–6 weeks and 1.4×10^7 B220$^+$ spleen B cells by 2 weeks. The peaks of the CD3$^+$ T and B220$^+$ B cells that were generated from 10^3 CLPs appeared ~7–10 days earlier than similar numbers of T and B cells derived from 10^3 HSCs. The changes of blood T and B cell numbers were almost synchronized with those in spleen T and B cells (data not shown). Neither Gr-1$^+$ nor Mac-1$^+$ myeloid progeny could be detected in either blood, bone marrow, or spleen in these experiments.

Figure 6 shows the sequential changes of CD4 and CD8 profiles of the thymic progeny derived from either 10^3 CLPs or 10^3 HSCs in the *RAG*$^{-/-}$ reconstitution assay. In normal thymus, immature CD4$^-$CD8$^-$ double negative cells rearrange *TCR* genes and proliferate to become CD4$^+$CD8$^+$ double positive (DP) cells, the DP cells receive a stringent selection determined by MHC-self-peptide and *TCR* interactions, and a minority of DP cells become CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ single positive (SP) cells (Akashi and Weissman, 1996). *RAG*$^{-/-}$ thymocytes cannot progress to the DP stage owing to the impairment of *TCR* gene rearrangements. As shown in Figure 6, the appearance of donor-type (Ly5.2) DP cells and SP cells was more than a week earlier in the thymus injected with 10^3 CLPs (upper panels) than those injected with 10^3 HSCs (lower panels). Thus, the CLP population

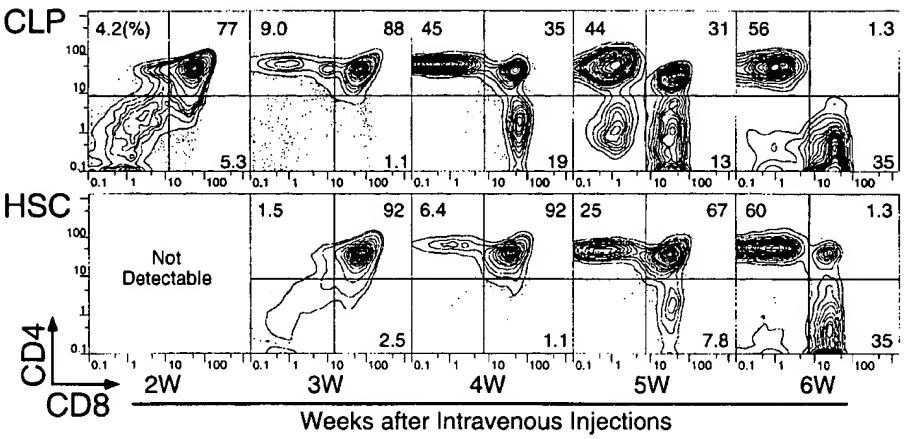

Figure 6. The Sequential Analysis of CD4 and CD8 Profiles of the Thymic Progeny after Reconstitution of *RAG*$^{-/-}$ (Ly 5.1) Mice with Syngeneic (Ly5.2) 10^3 CLPs or 10^3 HSCs

The appearance of CD4$^+$CD8$^+$ DP cells and CD4$^+$CD8$^-$ or CD4-CD8$^+$ SP cells from 10^3 CLPs preceded those from 10^3 HSCs by more than a week.

possessed a more rapid lymphoid differentiation capacity than the HSC population.

The Differentiation Potential of Lin$^-$IL-7R$^+$ Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells in Limit Dilution Analysis

We evaluated the lymphoid differentiation capacity of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells at limit dilution, where Poisson statistics would predict that, on average, a single cell can proliferate and differentiate into T or B cells. We competitively reconstituted the lethally irradiated mice with different numbers of the CLP (Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo}) cells with 2×10^5 host-type bone marrow. As shown in Figure 7A, approximately 1 in 22 cells can generate B cells after intravenous injection. In this experiment, we could detect donor-type T cell reconstitution in only 1 out of 18 mice that were injected with 35 CLP cells.

To circumvent the problem that T cell differentiation might fail if the cells did not home to the thymic microenvironment, we injected the CLPs directly into the thymus of sublethally irradiated congenic mice, as we did in some of the original assays to define HSCs (Spangrude et al., 1988). The frequency of cells that have the ability to respond to the thymic microenvironment was approximately 1 in 21 cells (Figure 7B). CD4 and CD8 profiles of the donor-derived cells show a normal distribution of CD4 and CD8 SP cells, and DP cells (Figure 7B).

We also checked the B cell differentiation capacity of the CLPs on the Whitlock-Witte-type in vitro culture (Whitlock and Witte, 1982; Muller-Sieburg et al., 1986), using the S17 stromal cell layers (Collins and Dorshkind, 1987). The CLP cells formed B220$^+$IgM$^+$ mature B cell-containing colonies after 14 days (Figure 7C). The frequency of cells that can read out differentiation into B lineage was approximately 1 in 6. Cumano et al. (1992) reported that the bipotent B cell/macrophage progenitor could differentiate into adherent macrophages as well as mature B cells in this culture condition in the presence of macrophage colony-stimulating factor (M-CSF). The addition of recombinant human M-CSF (25 U/ml) to the medium did not affect the frequency for B cell differentiation, and the cells did not give rise to adherent macrophages (data not shown).

Clonogenic Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells Can Give Rise to B, but Not to Myeloerythroid Colonies in Methylcellulose Culture

We further characterized the differentiation capacity of the CLP population in methylcellulose culture. First, we cultured the cells under the conditions for myeloid colony formation listed in Table 1. We have reported that the frequency of the cells that can respond to the methylcellulose cultures containing steel factor (SLF or c-Kit ligand), IL-3, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF) and erythropoietin were ~80% and ~20%–40% in long-term HSC and short-term HSC subsets, respectively (Morrison et al., 1996). However, the CLP (Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo}) cells did not form colonies in various cultures for myeloid colonies (Table 1).

It has been shown that B cell precursors in the bone marrow could form pre-B colonies in methylcellulose containing IL-7 (Suda et al., 1989). However, when we cultured 500 CLPs in the presence of IL-7 alone, only a few colonies were formed on day 7. These colonies were composed of pro-B (B220$^+$CD43$^+$IgM$^-$) and pre-B (B220$^+$CD43$^-$IgM$^-$) cells when analyzed on day 10 (data not shown). We considered the possibility that these earlier progenitors required earlier-acting cytokines. The addition of SLF and/or Flt-3/Flk2 ligand (FL) (Lyman et al., 1993; Hannum et al., 1994) to this culture condition significantly increased the frequency of colony-forming cells: ~20% of the CLP cells formed colonies in the presence of IL-7, SLF, and FL on day 7 (Table 1). These colonies were determined as pro-B/pre-B colonies on day 10 (Figure 7D). When we sorted different numbers of the cells into 96-well plates with methylcellulose containing IL-7, SLF, and FL, the frequency of the cells responding to this culture condition determined on day

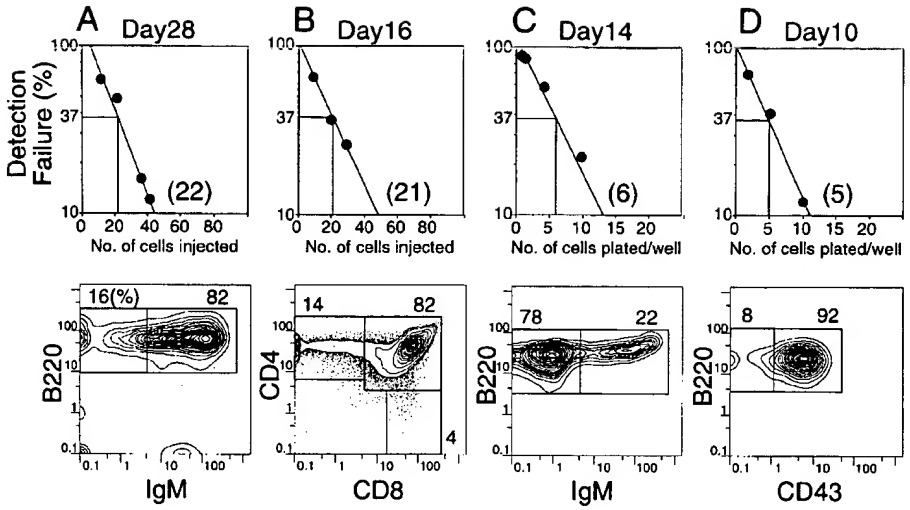

Figure 7. Results of Limit Dilution Analysis of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP Population

The differentiation potential of the cells was assessed by intravenous (A) and intrathymic (B) injections after 28 and 16 days, respectively, by culture on S17 stromal layers for 14 days (C), and by culture in methylcellulose containing SLF, IL-7, and FL for 10 days (D). The upper panels show the percentage of failure in detection of progeny from various numbers of cells under each condition. Numbers in parenthesis represent limiting numbers. Lower panels show surface phenotypes of the progeny under each condition. The differentiation outcomes on individual assays were determined as mature B cells in (A), all thymic T cell components in (B), mature B cells in (C), and pro-B/pre-B cells in (D). The surface phenotyping of a colony that grew in methylcellulose (D) was analyzed on day 10.

10 by the limit dilution analysis was also approximately 1 in 5 cells (Figure 7D).

Figure 8 shows the appearance of a day 3 colony derived from a single Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cell in the limit dilution analysis. The colonies were composed of 20–80 blastic cells. Interestingly, cells randomly changed their positions in methylcellulose along with cell divisions from day 1 to day 2, and the cells constituting the day 3 colony were dispersed, resembling the blast colonies reported by Nakahata and Ogawa (1982) that contained cells capable of self-renewal in vitro and of generating secondary myeloid colonies. We picked cells from the blastic day 3 colonies and replated them into the methylcellulose culture containing SLF, IL-3, IL-6, GM-CSF, M-CSF, and erythropoietin. However, we could not detect secondary myeloid colonies after replating in these conditions (data not shown).

To confirm that the cells in the day 3 colony possess differentiation potential for mature B cells in vivo, we picked the cells and injected them into lethally irradiated congenic mice with 2×10^5 host-type bone marrow cells. Four weeks after injection, B220$^+$IgM$^+$ mature B cell progeny could be detected in the spleen from 16 out of 18 injected mice (data not shown). Accordingly, virtually all clonogenic CLP cells in the methylcellulose culture can generate mature B cells, but not myeloid cells.

Single Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells Can Give Rise to Both T and B Cells

For the definition of CLPs, it was important to test

Table 1. Comparison of the Differentiation Potential of CLPs and HSCs in Methylcellulose in the Presence of Cytokines

No. of Day 7 Colonies/500 cells

	Cytokines Used for Myeloid Colonies						Cytokines Used for Lymphoid Colonies					
	SLF + M-CSF		SLF + IL-3 + IL-6 + GM-CSF + Epo		SLF + IL-3 + IL-6 + M-CSF + Epo		IL-7		SLF + IL-7		SLF + IL-7 + FL	
Cells	M	B	M	B	M	B	M	B	M	B	M	B
CLP: Lin$^-$IL-7R$^+$Sca-1^{lo} Thy-1$^-$c-Kit^{lo}	0	0	0	0	0	0	0	2 ± 1	0	34 ± 5	0	101 ± 8
HSC: Lin$^{-/lo}$IL-7R$^-$Sca-1^{hi} Thy-1^{lo}c-Kit^{hi}	1 ± 1	0	240 ± 6	0	154 ± 21	0	0	0	1 ± 1	0	2 ± 1	0

Different media and FCS were used for inducing either myeloid or B lymphoid colonies (see Experimental Procedures). Epo, Erythropoietin; M, myeloid colony included CFU-Mix, CFU-GM, BFU-E, and CFU-Meg (16); B, B lymphoid colonies consisted of pro-B and pre-B cells by phenotype. SLF alone could not stimulate colony-formation in both culture conditions.
Results are shown as mean ± S.D. of three independent experiments.

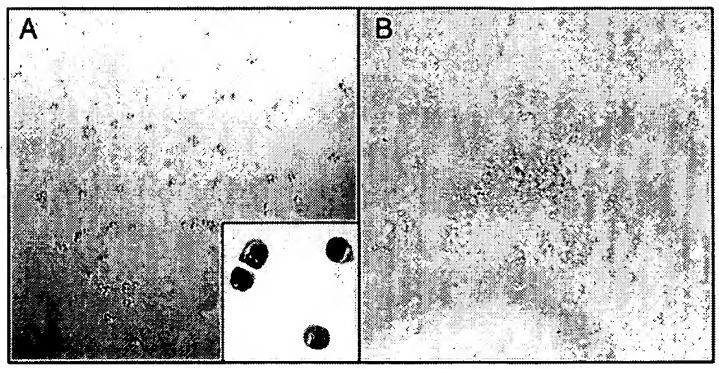

Figure 8. Colonies Derived from Single Lin$^-$ IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells in Methylcellulose in the Presence of IL-7, SLF, and FL

(A) The appearance of a day 3 colony that was derived from a single CLP on an inverted microscope and of the cells in the colony on Giemsa staining (right bottom). The colony has a dispersed distribution of blastic cells. (B) The appearance of a single CLP-derived day 7 colony. The colony is composed of B220$^+$IgM$^-$CD43$^+$ pro-B and B220$^+$IgM$^-$ CD43$^-$ pre-B cells (see Figure 7D).

whether a single Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cell could generate both T and B cells. We picked aliquots from the individual single cell-derived day 3 colonies that were grown in methylcellulose containing SLF, FL, and IL-7, and injected these cells intrathymically into sublethally irradiated congenic mice. In all cases, the remaining cells in the single cell-derived colonies continued to grow in methylcellulose and formed pro-B/pre-B cell colonies (Figure 9).

Sixteen days after injection, donor-derived T cells could be detected in 7 out of 20 injected thymuses. Representative data are shown in Figure 8. In 2 out of the 7 thymuses that developed donor-derived T cells, the cells showed normal thymic differentiation into both CD4$^+$ and CD8$^+$ SP T cells (case 1 in Figure 9, for example). The donor-derived cells contained both CD3$^+$ SP cells and CD3^{lo} DP cells. Neither myeloid cells nor B cells were detectable in the thymus. In the remaining 5

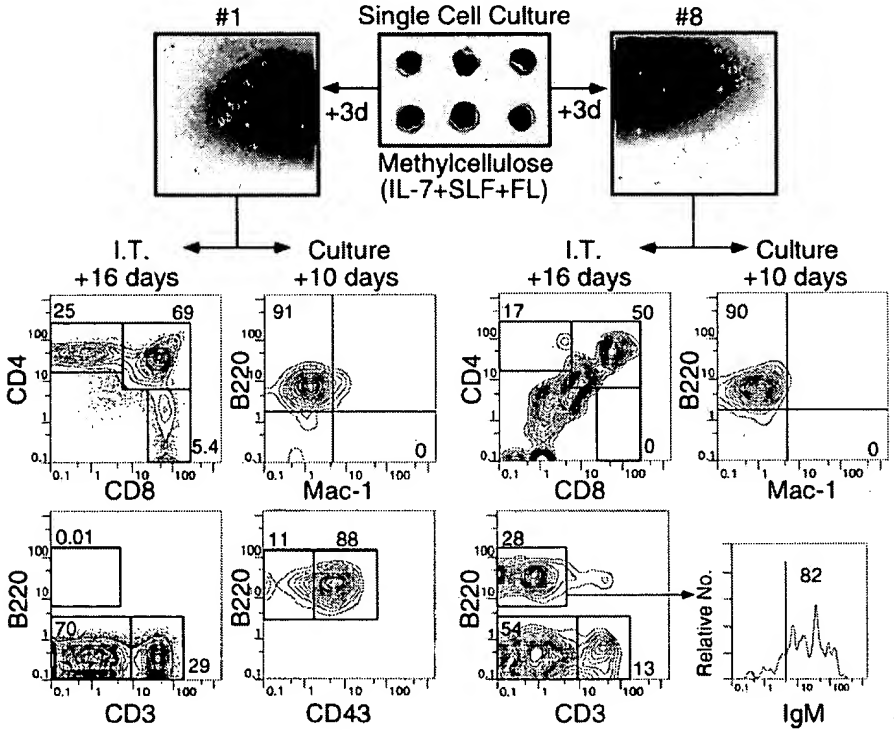

Figure 9. Clonal Analysis of B Cell and T Cell Progenitor Activity of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP Population

Morphology of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells from C57BL/Ka-Thy1.1 mice (Giemsa stain, top center). Day 3 colonies grew from individual CLP cells (cases 1 and 8) in methylcellulose in the presence of SLF, IL-7, and FL (photographs, top, left and right). The FACS plots show the surface phenotypes of thymic progeny after intrathymic injections (I.T.) of cells picked from single CLP cell-derived colonies and the surface phenotype of cells from the part of the colony that remained and was further cultured on the methylcellulose. Cells that were derived from a single CLP were able to differentiate into mature T cells (case 1) or both mature T and B cells (case 8) in the injected thymuses, and also differentiated into pro-B/pre-B cells in the methylcellulose in both cases.

cases (case 8 in Figure 9, for example), the progeny from a single cell consisted of B220$^+$ B cells as well as CD3$^+$ SP and CD3^{lo} DP cells. The B220$^+$ B cells were surface IgM$^+$ mature B cells. Myeloid cells were not detected. These data collectively indicate that at least some fraction of colony-forming cells in response to SLF, IL-7, and FL retained T-lineage as well as B-lineage differentiation potential after 3 days of the culture.

Discussion

Our data showed that the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo} c-Kit^{lo} population from bone marrow has prominent T and B cell reconstitution potential and contains CLPs that can give rise to at least both T and B cells. This population exhibited no or limited self-renewal activity. The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells could generate CD3$^-$NK1.1$^+$ NK cells in the spleen after in vivo injection (Figure 4). We have also observed the development of CD3$^-$NK1.1$^+$ NK cells from the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells in vitro after 2 weeks in culture on bone marrow stromal layers upon addition of IL-2 (Aguila et al., personal communication). Thus, the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells likely represent a clonogenic CLP population, though clonal studies for NK cells as well as T and B cells have not been done. Figure 10 summarizes our proposal for CLPs in lymphoid development sequences from HSCs.

The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells completely lack differentiation capacity to myeloid lineages both in vivo and in vitro. First, neither Gr-1$^+$ nor Mac-1$^+$ myeloid cells could be detected from 1 to 24 weeks after reconstitution in the series of in vivo reconstitution analysis, and 2 $\times$ 10^3 CLPs could not give rise to either day 8 CFU-S or donor-derived myeloid (Mac-1$^+$ or Gr-1$^+$) cells in bone marrow 8 days after injection (data not shown). Second, we did not detect myeloid cells, including adherent macrophages, during the culture over 21 days on S17 stromal layers in the presence or absence of M-CSF (data not shown), although the HSCs and the "bipotent" B cell/macrophage precursors reportedly give rise to both B and myeloid cells in this condition (Cumano et al., 1992; Morrison et al., 1996). Third, although HSCs formed a variety of myeloid colonies in methylcellulose in the presence of SLF, IL-3, IL-6, GM-CSF, and erythropoietin in our hands (Morrison et al., 1996), the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells did not form myeloid colonies in the culture. The cells constituting day 3 colonies grew in the presence of IL-7, SLF and FL did not give rise to secondary myeloid colonies in the presence of myeloid-directed cytokines in a replating experiment, whereas they could give rise to only T and B cell progeny after in vivo transfer. These data indicate that the differentiating potential of the CLP population is strictly limited to T, B, and NK lineages. Accordingly, this population represents the earliest known stage of lymphoid development distinct from HSCs.

The earliest thymic precursor population could generate B, NK, and LD cells (Wu et al., 1991a; Ardavin et al., 1993; Matsuzaki et al., 1993). The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP population likely gives rise to LD cells as well as T, B, and NK cells, because ~5% of

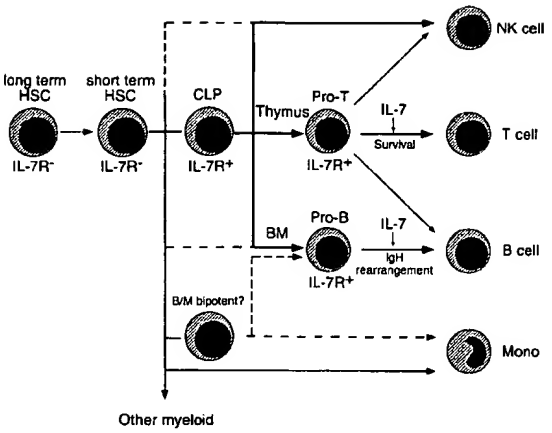

Figure 10. The Proposed Differentiation Sequences from HSCs to Lymphoid Cells in Adult Mice

The HSC population including both long-term and short-term HSCs does not express IL-7R. They might express IL-7R heterodimer immediately after the commitment to lymphoid lineages and become the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLPs that are able to generate all lymphoid classes. The CLPs differentiate into pro-B cells in bone marrow (BM), whereas the CLPs that successfully reach thymic microenvironment can read out T cell differentitation. The earliest thymic precursor (pro-T) population might include a small fraction of CLPs that have just homed to the thymus and still possess differentiation potential into B and NK cells (Wu et al., 1991a; Matsuzaki et al., 1993). IL-7R is expressed in both T cell and B cell progenitors, but IL-7 plays different roles in T- and B cell development in vivo; the principal role of IL-7 in T cell development is to maintain cell survival through up-regulating (at least) Bcl-2 (Akashi et al., 1997), but that in B cell development is other than maintenance of cell survival (Kondo et al., 1997). The principal role of IL-7 in B lymphopoiesis is probably to promote rearrangement of *IgH* genes (Corcoran et al., 1996). Some of lymphoid cells may be developed directly from short-term HSC or multipotent progenitors. It is still unclear that B cells can also be derived from bipotent B cell/myeloid progenitors (Cumano et al., 1992) in vivo (see Discussion).

the cells differentiate into morphologically determined dendritic cells after 7-day culture in methylcellulose containing SLF, IL-7, TNF-α, plus IL-1β (K. A. et al., unpublished data). On the other hand, the earliest thymic precursors did not give rise to colonies in the methylcellulose cultures containing IL-7, SLF, and/or FL (K. A. et al., unpublished data). The surface phenotype of the thymic precursor was different from that of the CLP population in the bone marrow (Figure 2). Furthermore, in contrast to the proven potency of the CLP cells in our competitive reconstitution assay (e.g., the 2 $\times$ 10^3 cells shown in Figure 2A), 4.5 $\times$ 10^3 thymic precursors from the same C57BL strain we used here gave rise to little or no progeny after intravenous injection to lethally irradiated congenic mice with 5 $\times$ 10^4 recipient-type bone marrow cells (Wu et al., 1991a), and the injection of large numbers (1–3 $\times$ 10^4) of cells was required to detect the B and NK cell progeny (Wu et al., 1991a; Matsuzaki et al., 1993). Thus, the thymic precursors and the CLP populations differ in surface phenotype, in frequency, in proven clonogenic capacity, and in their respective "burst" size. Based on these data, it is possible that the earliest thymic precursor population may be heterogeneous; this population may contain rare CLP

cells or a small number of oligopotent progenitors for B, NK, and/or LD cells as well as T cell–committed progenitors.

The IL-7R$^+$ CLP cells respond to IL-7 in cultures by expansion, at least for several cell doublings, retaining their lymphoid-specific differentiation capacity. Does the IL-7R play a role for generating these cells from more primitive HSC and/or progenitor cells? This seems unlikely, since the frequency of the phenotypically determined CLP population in γ_c-deficient mice is comparable to that in normal mice (M. K. et al., unpublished data). On the other hand, the γ_c-deficient mice showed reduced frequencies of early pro-B and pro-T cells (Kondo et al., 1997). IL-7R has been shown to mediate pivotal signals for promoting *IgH* rearrangement for B cell precursors (Corcoran et al., 1996) and survial for T cell precursors (Akashi et al., 1997). Thus, it could be that the expression of functional IL-7R complexes represents the initial cellular change in the differentiation from HSCs to CLP cells. It is important to clarify intracellular events linked to lymphoid commitment that lead to IL-7R expression. The expression of IL-7R may enable CLPs to respond to IL-7 in the context of local stromal microenvironments to give rise to the specified lymphocyte classes and subsets.

The generation of T and B cells from the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP population is rapid compared to that from HSCs (Figures 5 and 6). Accordingly, it is reasonable to postulate that the CLP population from the bone marrow could represent a transition state between the known HSC (IL-7Rα^-) subsets (Spangrude et al., 1988; Morrison and Weissman, 1994; Osawa et al., 1996) and the earliest thymic precursors (Wu et al., 1991; Matsuzaki et al. 1993) that are IL-7Rα^+ (M. K. et al., unpublished data) (Figure 10). We have not yet critically assessed whether the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} stage is a required intermediate for lymphoid differentiation. In the bone marrow, Antica et al. (1994) have reported that a Lin$^-$Thy-1^{lo}HSA$^+$Sca-2$^+$ population may be the latest precursor for generating the earliest thymic precursor. This population represented ~0.25% of bone marrow cells (~10-fold more than our population) and retained pluripotent differentiation capacity. It is possible that T and B cell development may occur directly from some pluripotent progenitors or HSC subsets that induce clonogenic cells that retain the differentiation capacity to myeloid cells (Antica et al., 1994; Morrison and Weissman, 1994).

Several reports revealed a "close" relationship between the development of B and monocyte lineages: Klinken et al. (1988) demonstrated that the transfection of v-*raf* to B cell lines established from Eμ-*myc* transgenic mice could convert them into macrophages that maintained the identical rearrangement of immunoglobulin heavy chain (*IgH*) genes. The clonal analysis of human leukemias/lymphomas with B cell/myelomonocytic phenotypes (Mirro et al., 1986; Akashi et al., 1991; 1993) supports the possible existence of B cell/myelomonocytic bipotent progenitors, though the transformation event itself may perturb the normal differentiation process. In normal hematopoiesis, Hirayama et al. (1992) reported that B cells could develop from myeloid colony-forming cells from mouse bone marrow, but they later showed that some of these cells retained the capacity for T cell differentiation (Hirayama and Ogawa, 1995), suggesting that the colony-forming cells might be pluripotent. A population of cells isolated from fetal liver gives rise to both B cells and macrophages from single cells in vitro (Cumano et al., 1992). However, T cell developmental potential was not tested in that study. It remains unclear whether B cells actually develop from "bipotent" B cell/myeloid or macrophage precursors in vivo.

Although the CLP population presented here has distinct phenotypes discernible from HSCs and completely lacks myeloid differentiation potential in a variety of assays both in vivo and in vitro, we should still take the limitation of the assays into account; the CLPs may not home suitable microenvironments for myeloid differentiation after intravenous injection in vivo or may be able to develop myeloid cells by the application of alternate, yet-to-be-developed in vitro assays. This warrants further analyses of the cellular characteristics of this population, including the expression of other known or unknown cytokine receptors and of transcription factors that are related to myeloid or lymphoid commitment. It is also of interest to determine the CLP population in fetal hematopoiesis to clarify fetal developmental processes of lymphoid cells including TCR/γ/δ T cells (Ikuta et al., 1990). Kawamoto et al. (1997) reported that T/B bipotent prognitors were undetectable in either the Lin$^-$c-Kit$^+$Sca-1$^+$ population enriched for HSCs or the Lin$^-$c-Kit$^+$Sca-1$^-$ progenitor fractions in fetal liver, determined by fetal thymic organ cultures of single cells in the presence of SLF, IL-3, and IL-7. However, results of lineage outcomes may reflect the bias of lineage readout in the assay system they used and/or the stochastic nature of lineage commitment of HSCs and progenitors. The isolation of a distinct population that possessed lymphoid-restricted differentiation potential in fetal liver will be a prerequisite for determining the potential role of CLPs in fetal lymphopoiesis.

The generation of T and B cells from the CLP population was prominent. The injection of as few as 10^3 CLPs could considerably restore the numbers of spleen B and T cells in the immunodeficient *RAG-2*$^{-/-}$ mice as early as 2 weeks and 4 weeks after injection, respectively. Accordingly, the transplantation of the CLP population (or its human counterpart) might be able to rapidly correct the lymphoid deficiencies that are found in various immunocompromised situations. The transduction of genes into HSCs is currently difficult by using retrovirus vectors, because the long-term HSCs are mainly quiescent (Morrison and Weissman, 1994). Since a considerable fraction of CLPs is cycling and the CLPs could respond to defined cytokines in vitro, transduction of genes into the CLPs might be efficient, and this might benefit future gene therapy targeted for lymphoid cells.

Thus, our data provide direct evidence for the existence of a mouse clonogenic CLPs in vivo. Further characterization of this population could help clarify events in early lymphoid commitment. It is possible that a counterpart population in humans may also be isolated by using IL-7R as a marker as well as CD10, CD34, c-Kit, and/or Thy-1 (Galy et al., 1995). The identification of the

human CLPs might be useful for establishing lymphoid-restricted therapeutic approaches for various immune disorders.

Experimental Procedures

Mouse Strains

The congenic strains of mice, C57BL/Ka-Thy1.1 (Ly5.2) and C57BL/Ka-Thy1.1-Ly5.1 mice were used for the reconstitution assays. The strains differed only at the *Ly5* allele, and this difference made it possible to easily detect donor-derived cells generated from hematopoietic progenitors. C57BL/6 *RAG-2*$^{-/-}$ (Ly5.1) mice were generated by crossing C57BL/6 *RAG-2*$^{-/-}$ (Ly5.2) mice with C57BL/6-Ly5.1 mice. The C57BL/6 *RAG-2*$^{-/-}$ (Ly5.1) mice were used as recipients. Mice were bred and maintained in the animal care facility at Stanford University School of Medicine and were used at 4–10 weeks of age.

Cell Staining and Sorting

For sorting HSC and CLP populations, bone marrow cells were stained with unconjugated rat antibodies specific for lineage (Lin) markers (CD3, KT31.1; CD4, GK1.5; CD8, 53-6.7; B220, 6B2; Mac-1, M1/70; Gr-1, 8C5; and TER119). Lin$^+$ cells were partially removed with sheep anti-rat IgG-conjugated immunomagnetic beads (Dynabeads M-450, Dynal A. S., Oslo, Norway), and the remaining cells were stained with Cy5-PE (Tricolor)-conjugated goat anti-rat IgG polyclonal antibodies (Caltag, Burlingame, CA). After incubation with rat IgG (Sigma, St. Louis), cells were stained with FITC-conjugated anti-Thy-1.1 (19XE5), Texas red–conjugated anti-Sca-1 (E13-161-7) and APC-conjugated anti-c-Kit (2B8) monoclonal antibodies. IL-7Rα was stained with biotinylated anti-IL-7Rα antibody (A7R34) and was visualized by PE-conjugated streptavidin (Caltag). For the analysis of the pre-B and pro-B populations, bone marrow cells were stained with biotinylated anti-Gr-1 and TER119, PE-conjugated anti-CD43 (S7) (Pharmingen), Texas red–conjugated polyclonal anti-mouse IgM (Caltag), and APC-conjugated anti-c-Kit. Cells were then stained with Cy5-PE (RED670)–conjugated streptavidin (GIBCO BRL, Gaithersburg, MD). For the thymic progenitor population, thymocytes were stained with anti-CD4 and anti-CD8, followed by Texas red–conjugated anti-rat IgG polyclonal antibodies (Caltag). After incubation with rat IgG, cells were stained with PE-conjugated anti-CD3, APC-conjugated anti-c-Kit, and biotinylated anti-CD25 antibody (7D4, Pharmingen). Anti-CD25 was visualized by Cy5-PE-conjugated streptavidin. FITC-conjugated antibodies for HSA (CD24; M1/69), CD43, and CD44 (IM7) were purchased from Pharmingen. For cell cycle analysis, cells were first incubated with 10 μg/ml Hoechst 33342 (Sigma) as described previously (Morrison and Weissman, 1994). These cells were sorted or analyzed using a highly modified triple laser (488-nm argon laser, 599-nm dye laser, and UV laser) FACS (FACS Vantage, Becton Dickinson Immunocytometry Systems, Mountain View, CA). Progenitors were purified by sorting and then resorting to obtain precise numbers of cells that were essentially pure for the indicated surface marker phenotype. In the limit dilution assays and single-cell clonogenic assays, the resort was performed by using a carefully calibrated automatic cell deposition unit (ACDU) system (Becton Dickinson). This system deposited a specific number of purified cells onto either methylcellulose medium, S17 stromal cell cultures or HBSS in 96-well plates. In most cases, we reconfirmed the specific number of cells sorted in each well under an inverted microscope.

Evaluation of *TCRβ* and *IgH* Gene Rearrangement Status

PCR analysis was used for detecting rearrangement of the *TCRβ* and *IgH* genes as described previously (Mebius et al., 1997). Briefly, 2000 sorted cells were incubated at 94°C for 10 min in 0.5 % Tween D in PCR buffer prior to PCR reaction. Primer sequences used for *TCRβ* gene in first PCR are 5′-TAGGCACCTGTGGGGAAGAAAC-3′ (Db2.1 ext) and 5′-TGAGAGCTGTCTCCTACTATC-3′ (Jb2.7 ext). Aliquots (1 of 5) were further amplified with internal primer pair, 5′-GTA TCACGATGTAACATTGTG-3′ (Db2.1 int) and 5′-GGAAGCGAGAGAT GTGAATC-3′ (Jb2.7 int). The primers for detecting *IgH* rearrangement were 5′-GACTAAGCGGAGCACCACAG-3′ (DQ52) and 5′-CTCTCAGCCGGCTCCCTCAGGG-3′ (JH4#2). The samples were denatured (94°C, 30 s), annealed (58°C, 2 min), and extended (72°C, 3 min) for 35 cycles. Amplified DNA was subjected to electrophoresis in 1.2% agarose gel.

In Vivo and In Vitro Assays to Determine Differentiation Potential of CLPs

For reconstitution assays, the purified common lymphoid progenitors (CLPs) were injected into the retroorbital venous sinus of lethally irradiated (920 rad) congenic mice, which differed only at the *Ly5* allele, together with 2×10^5 host Ly5–type bone marrow cells. The levels of donor-derived blood nucleated cells were monitored as described elsewhere (Morrison and Weissman, 1994). When *RAG-2*$^{-/-}$ mice were used as recipients, purified cells were intravenously injected into *RAG-2*$^{-/-}$ mice after irradiation at 400 rad. Intrathymic injection was performed by directly injecting cells into thymuses of mice that had been irradiated (600 rad) as described previously (Akashi and Weissman, 1996, Akashi et al., 1997).

To support the formation of myeloid colonies, progenitors were cultured in an alpha-Modified Eagle Medium (αMEM)–based methylcellulose media (Methocult M3100; StemCell Technologies, Vancouver, Canada) that was supplemented with 30% fetal bovine serum (FBS), 1% bovine serum albumin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. Cytokines such as mouse SLF (100 ng/ml; provided by Immunex), mouse IL-3 (30 ng/ml; Genzyme, Cambridge, MA), mouse IL-6 (10 ng/ml; Genzyme), mouse GM-CSF (10 ng/ml; Genzyme), human M-CSF (25 U/ml; Genzyme), and human erythropoietin (1 U/ml) were added at the start of the culture. To examine lymphoid colony formation, we used Iscove's Modified Dulbecco's Medium (IMDM)–based Methocult M3630 (StemCell Technologies) containing human IL-7 (10 ng/ml) supplemented with SLF (100 ng/ml) and/or human FL (20 ng/ml; Genzyme). Progenitors were also cultured on irradiated (2000 rad)-S17 stromal cell layers in 96-well plates with RPMI 1640 medium containing 10% FBS (Gemini Bioproducts, Calabasas, CA), SLF (100 ng/ml), and human IL-7 (10 ng/ml; gift from Sanofi). All cultures were incubated at 37°C in a humidified chamber under 7% CO_2.

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EXHIBIT 2

Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines

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The primary role of cytokines in haemato-lymphopoiesis is thought to be the regulation of cell growth and survival[1–3]. But the instructive action of cytokines in haematopoiesis has not been well addressed[4]. Here we show that a clonogenic common lymphoid progenitor[5], a bone marrow-resident cell that gives rise exclusively to lymphocytes (T, B and natural killer cells), can be redirected to the myeloid lineage by stimulation through exogenously expressed interleukin (IL)-2 and GM-CSF (granulocyte/macrophage colony-stimulating factor) receptors. Analysis of mutants of the β-chain of the IL-2 receptor revealed that the granulocyte- and monocyte-differentiation signals are triggered by different cytoplasmic domains, showing that the signalling pathway(s) responsible for these unique developmental outcomes are separable. Finally, we show that the endogenous myelomonocytic cytokine receptors for GM-CSF and macrophage colony-stimulating factor (M-CSF) are expressed at low to moderate levels on the more primitive haematopoietic stem cells, are absent on common lymphoid progenitors, and are upregulated after myeloid lineage induction by IL-2. We conclude that cytokine signalling can regulate cell-fate decisions and propose that a critical step in lymphoid commitment is downregulation of cytokine receptors that drive myeloid cell development.

IL-15 has been proposed to be a critical cytokine for natural killer (NK) cell development[6–8]. The receptor for IL-15 shares two subunits with the IL-2 receptor (IL-2R), namely the common γ (γ_c) and IL-2Rβ chains[9,10]. Given this conservation of subunits, we decided to determine whether IL-2 receptor signalling can induce NK cell development from common lymphoid progenitors (CLPs). CLPs express the γ_c but not the IL-2Rβ chain (data not shown). Human IL-2Rβ can couple with mouse γ_c chain to form a functional IL-2 receptor that responds exclusively to human IL-2 (hIL-2)[11,12]; therefore, we cultured CLPs from mice transgenic for the human IL-2Rβ chain[13] on OP9 bone marrow stromal cells[14] in the presence of hIL-2. IL-2Rβ transgene expression is driven by the MHC class I promoter and cell-surface expression was observed in nearly all haematopoietic cells, including haematopoietic stem cells (HSCs) and CLPs (data not shown). After 10 days the culture contained an unexpectedly large number of Gr-1$^+$ granulocytes and monocytes (GM lineage cells, Fig. 1a) in addition to CD19$^+$ B cells and NK1.1$^+$ NK cells (see Fig. 2). Under these same culture conditions, CLPs from wild-type mice (C57BL/Ka-Thy1.1) gave rise to B and NK cells but never to GM cells (data not shown). In the absence of hIL-2, CLPs from IL-2Rβ transgenic mice are still lymphoid-committed, even in the presence of the myelo/monocytic cytokines IL-3, GM-CSF and M-CSF (Fig. 1a). When we carried out colony-forming assays in methylcellulose, we found that in addition to CLPs, pro-T cells from the thymus of IL-2Rβ transgenic mice could also convert to the myeloid lineage when stimulated with hIL-2 (Fig. 1b). However, IL-2Rβ-expressing pro-B cells from the bone marrow did not give rise to GM cells under any culture conditions. These data indicate that CLPs and pro-T cells may have a latent GM lineage differentiation program that can be initiated by signalling through the reconstituted IL-2 receptor. It is important to note that there is a 2-day window after which cultured CLPs lose this IL-2-induced GM lineage differentiation potential and 'irreversibly' commit to the lymphoid lineage (Fig. 1a). Conversely, the lineage converting effect of IL-2 is complete in 2 days and can be withdrawn without any deleterious effect on the conversion process under GM growth

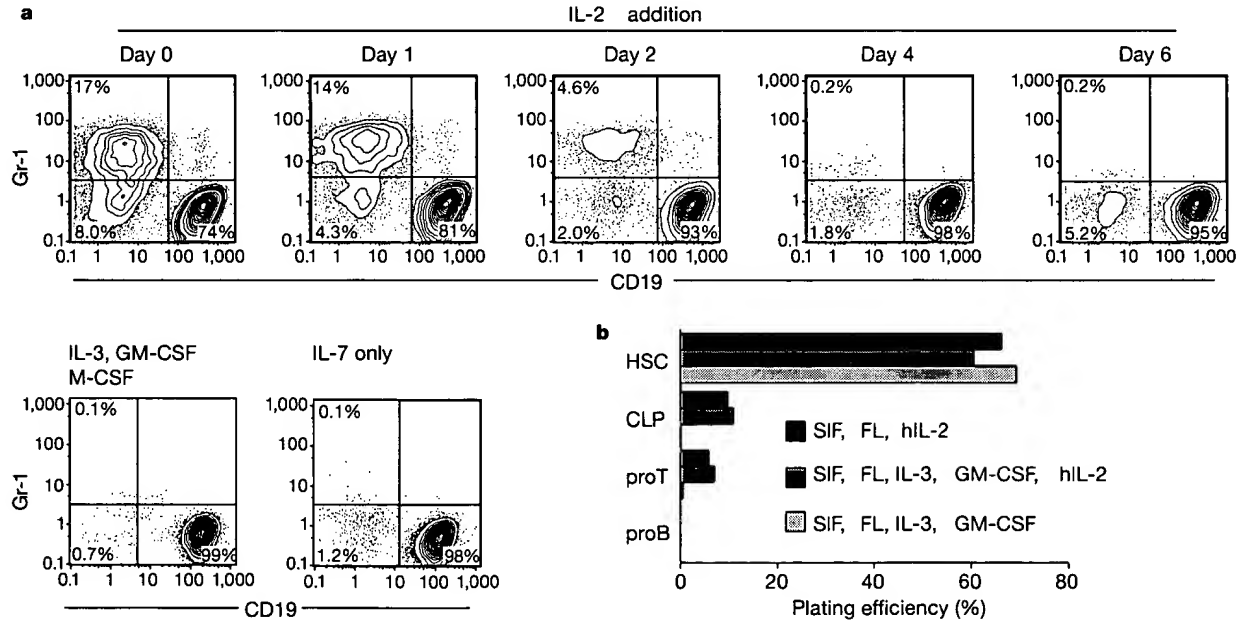

Figure 1 Granulocyte/macrophage differentiation from CLPs of IL-2Rβ transgenic mice. **a**, Flow cytometric analysis of cell populations from 40 CLPs of IL-2Rβ transgenic mice after stromal cell culture with OP9 in the presence of IL-7 (10 ng ml^{-1}) for 10 days. Human IL-2 (25 ng ml^{-1}) was added to the culture at the indicated time points (top row). No Gr-1$^+$ cells were observed when GM-CSF (10 ng ml^{-1}), IL-3 (30 ng ml^{-1}) and M-CSF (25 U ml^{-1}) were added into the culture (bottom row, left). **b**, Colony-forming activity of cells from IL-2Rβ transgenic mice. FACS-sorted cells were cultured in methylcellulose for 5–8 days in the presence of various cytokines indicated in the figure. All colonies were morphologically GM colonies. Results shown are means of more than three independent cultures.

conditions (data not shown). In addition, we found that the cell-fate conversion was limited to the GM lineage, as no colonies grew under erythroid culture conditions with IL-2.

There are two possible explanations for the GM lineage readout that we observed in our experimental system. First, IL-2 receptor signalling may be rescuing from apoptosis a myeloid-committed population of cells that co-purifies with CLPs. However, we observed no lineage conversion from CLPs expressing the anti-apoptotic protein Bcl-2 (ref. 15) when cultured under GM growth conditions (data not shown). Second, IL-2 receptor signalling may be actively promoting the development of GM lineage cells from lymphoid-committed progenitors. To test this hypothesis, we examined the trans-differentiation of CLPs from IL-2Rβ transgenic mice at the clonal level.

Limiting dilution analysis[16] revealed that the GM cell readout frequency of CLPs from IL-2Rβ transgenic mice was 1 event per 4 cells when IL-2 was added at day 0 (Table 1). In the absence of IL-2, B-cell readout frequency of CLPs was 1 out of 10. However, B-cell development dropped to the level of 1 in 40 when IL-2 was added at day 0, suggesting that GM lineage conversion had occurred at the expense of lymphoid development.

Next, we clone-sorted CLPs from IL-2Rβ transgenic mice into 96-well plates and added IL-2 at day 0, 2 or 4 of the culture. Consistent with the results of the limiting dilution assay above, B-cell readout was severely blocked when IL-2 was added into the culture at day 0 or 2, when GM lineage conversion can be initiated (data not shown; see Supplementary Information). More importantly, about 20% of scorable wells had both GM and lymphoid lineage cells when IL-2 was added at day 2 (Fig. 2; see GM/B, GM/NK and GM/B/NK). This directly shows that IL-2 receptor signalling can drive myeloid lineage commitment from lymphoid committed progenitors. In addition to this observation, ~5% of pro-T-cell-derived GM colonies from IL-2Rβ transgenic mice (Fig. 1b) had rearranged T-cell receptor loci, an event seen only in lymphoid lineage cells[17] (A.G.K., M.K. and I.L.W., manuscript in preparation). These results show that early lymphoid committed progenitors maintain a latent GM lineage differentiation activity that can be triggered by signalling through an exogenously expressed cytokine receptor.

To assess the molecular mechanisms that drive GM lineage conversion in lymphoid-restricted progenitors, we used retrovirus-mediated gene transfer to introduce wild-type and mutant human IL-2Rβ chains[18] into CLPs from wild-type mice and

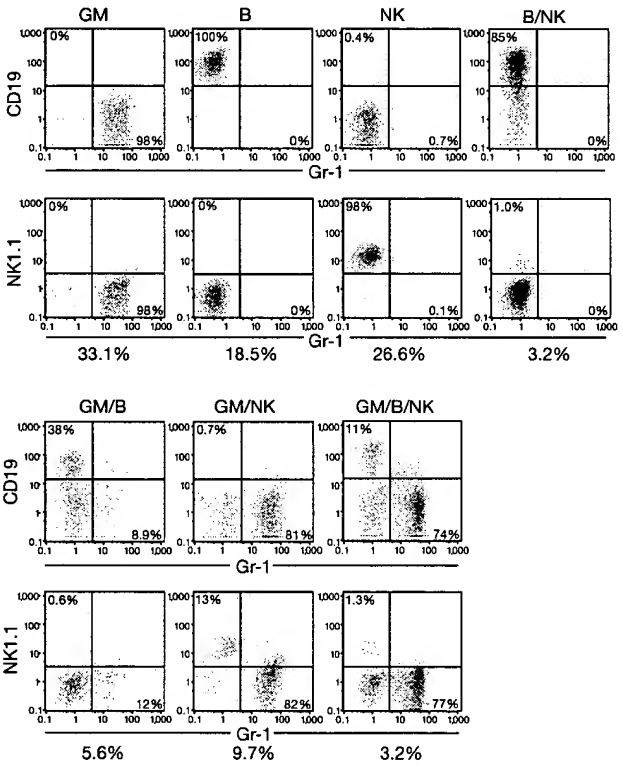

Figure 2 Clonogenic trans-differentiation activity of CLPs from IL-2Rβ transgenic mice. Single ACDU-sorted CLPs from IL-2Rβ transgenic mice were each cultured on an OP9 stromal cell layer in each well of 96-well plates for 7 days in the presence of SIF, FL and IL-7. Human IL-2 was added at day 2 of culture. Positive readout wells were determined by microscopic observation (124 wells out of 576 wells, 21.5%). The readout pattern was determined by flow cytometric analysis. The frequency of wells with each differentiation pattern is shown as a percentage of total positive wells under each FACS plot.

Table 1 Limiting number of IL-2Rβ transgenic CLPs for each lineage readout

	Day of culture when human IL-2 was added		
Readout cells	Day 0	Day 4	–
GM cells	4	>500	∞
B cells	40	11	10
NK cells	27	24	150

Between 1 and 40 CLPs from IL-2Rβ transgenic mice were cultured on an OP9 stromal cell layer in the presence of IL-7. Human IL-2 was added at indicated time points. The graphs used to obtain the limiting numbers are given in the Supplementary Information.

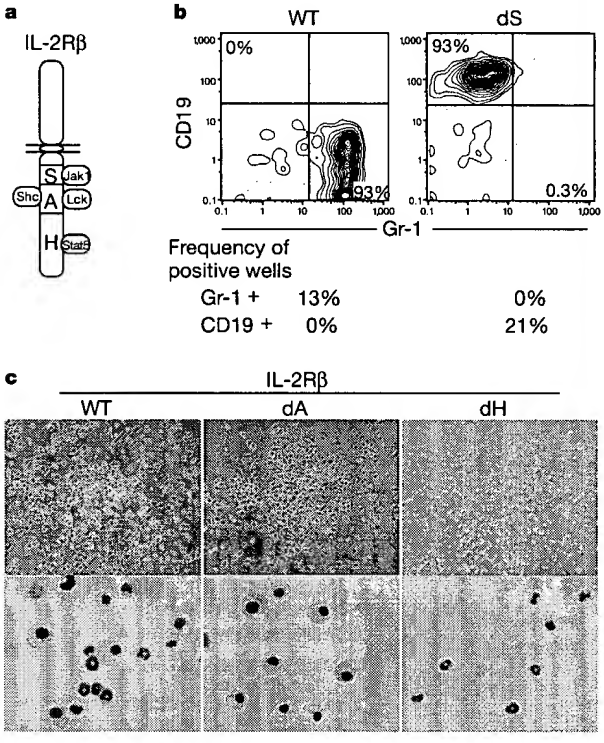

Figure 3 Induction of myeloid cells from CLPs of wild-type mice. **a**, Representation of IL-2Rβ structure. See Supplementary Information for diagram of mutants. **b**, CLPs from C57BL/Ka-Thy1.1 mice were infected with either wild-type IL-2Rβ (WT) or the dS mutant retrovirus. After culture in the presence of IL-2 for 48 h, GFP$^+$ cells were sorted and cultured on OP9 cells for 6 days in the presence of IL-3 and GM-CSF. Gr-1$^+$ cells were morphologically granulocytes and monocytes. **c**, Lineage conversion induced through IL-2Rβ mutants. Wild-type, dA and dH mutants of IL-2Rβ chains were introduced in CLPs from wild-type mice by retroviral infection. After pre-culture as in **b**, GFP$^+$ cells were further cultured in methylcellulose for 5–8 days in the presence of SIF, IL-3 and GM-CSF (see Supplementary Information). Colony type was determined under microscopic observation and confirmed by cytospin. Over 95% of cells in the colonies derived from CLPs with the dA and dH mutants were macrophages and granulocytes, respectively, by differential cell count.

analysed their development *in vitro*. All retroviral constructs contained a green fluorescent protein (GFP) complementary DNA driven by an internal ribosome entry site (IRES) for the purpose of identifying infected cells. After infection, we cultured cells for 48 h in the presence of hIL-2 to induce GM lineage conversion. We then sorted GFP^+ CLPs, and cultured them on stromal cells for 6 days in the absence of hIL-2. CLPs expressing the wild-type IL-2Rβ chain gave rise to $Gr\text{-}1^+$ cells but not to $CD19^+$ B cells (Fig. 3b). In contrast, CLPs expressing the non-functional dS mutant[18], which lacks the S region of the cytoplasmic tail of IL-2Rβ (Fig. 3a), differentiated into B cells, but not into GM lineage cells. This shows that signals emanating from the cytoplasmic tail of IL-2Rβ are critical for GM lineage conversion of CLPs.

To characterize further the signalling requirements for GM lineage conversion in CLPs, we analysed two additional IL-2Rβ mutants, dA and dH[18], which lack the A and H regions of the cytoplasmic domain of IL-2Rβ chain, respectively (Fig. 3a). The A region is necessary for recruitment of Shc and Src tyrosine kinases, such as Lck and Fyn; the H region is crucial for Stat5 activation[19]. Infection and pre-culture of CLPs in the presence of hIL-2 was performed as before, and GFP^+ cells were then sorted into methylcellulose culture medium that supports myeloid differentiation. After 5–7 days of culture, CLPs expressing wild-type IL-2Rβ formed GM colonies at 5–10% plating efficiency (Fig. 3c), whereas CLPs expressing the non-functional dS mutant did not form detectable colonies (data not shown). Notably, CLPs expressing the dA mutant formed exclusively macrophage colonies, whereas those with the dH mutant formed only granulocyte colonies (Fig. 3c). Thus macrophage and granulocyte differentiation are induced by different cytoplasmic regions of the IL-2Rβ chain, and the signal transduction cascades that drive granulocyte and macrophage differentiation are separable.

The known signalling pathways activated by the IL-2 receptor have significant overlap with those activated by the receptors for erythropoietin (Epo) and IL-7. For example, Shc is phosphorylated in response to stimulation with either Epo or IL-2, whereas all three cytokines activate the transcription factor Stat5 (refs 19, 20). However, Epo stimulation did not induce GM lineage conversion in CLPs expressing retrovirally encoded EpoR (Fig. 4a). Likewise, IL-7 did not induce GM lineage conversion in CLPs through either endogenously or exogenously expressed IL-7 receptor. These data, together with the evidence obtained with the dA and dH mutants of IL-2Rβ, suggest that unknown signal transduction pathways are involved in the initiation of GM lineage conversion in CLPs. They also show that GM lineage conversion in CLPs is the result of the activation of specific signalling cascades for differentiation, not just the supportive effect of cytokines for cell proliferation and survival.

What is the significance of IL-2 induced GM lineage conversion of CLPs expressing IL-2Rβ? One possibility is that the IL-2 receptor mimics other cytokine receptors that support GM lineage differentiation, such as the GM-CSF receptor. To examine this possibility, we transduced wild-type CLPs with a retrovirus that expresses a functional human GM-CSF receptor[21] and cultured these cells in the presence of human GM-CSF for 2 days. After infection, the cells were then replated in methylcellulose under myeloid culture conditions. After 5–7 days in culture, GM colonies were formed (Fig. 4b) in transduced but not in wild-type CLPs. Expression of the exogenous human GM-CSF receptor in the colony-forming cells was confirmed by polymerase chain reaction with reverse transcription (RT–PCR) (Fig. 4b). This result prompted us to examine the expression of the GM-CSF and M-CSF receptors in CLPs from IL-2Rβ transgenic mice in response to IL-2. In the absence of IL-2, CLPs expressed neither the GM-CSF receptor α-chain nor the M-CSF receptor (Fig. 4c); however, expression of these two molecules was strongly induced in the presence of IL-2 after 48 h. HSCs, which give rise to both lymphoid and myeloid lineages, expressed low to moderate levels of the GM-CSF and M-CSF receptors. From these data, we propose that one of the earliest events in lymphoid lineage commitment is downregulation of cytokine receptors whose function is to drive and support GM lineage differentiation.

Significantly, some acute myeloid leukemia (AML) cells have rearrangements of immunoglobulin and T-cell receptor genes together with myeloid-specific antigens[22–24]. The developmental plasticity of lymphoid lineage progenitors revealed here may help to explain such 'lineage infidelities'. Finally, the finding that cytokine receptor signalling can instructively drive lineage commitment will allow us to begin to elucidate the molecular mechanisms that regulate haematopoietic cell differentiation. □

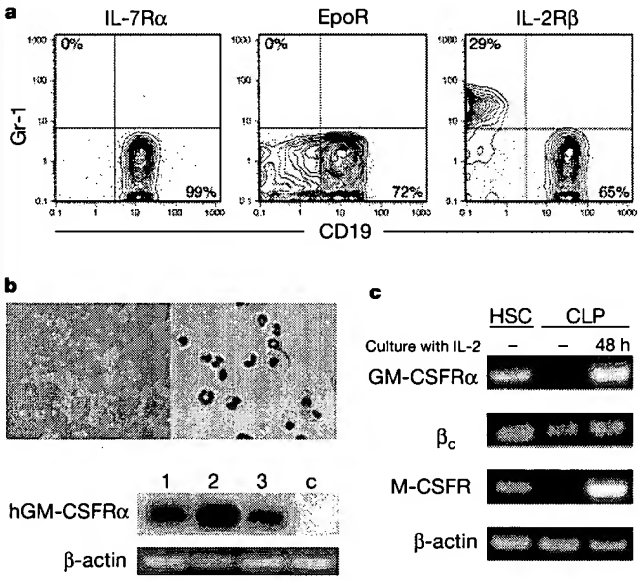

Figure 4 Granulocyte/macrophage induction in CLPs is initiated by stimulation with GM-CSF. **a**, Epo stimulation does not induce GM lineage conversion in CLPs. IL-7Rα (left), EpoR (middle) and IL-2Rβ (right) were introduced into CLPs from C57BL/Ka-Thy1.1 mice by retroviral vectors. For infection and pre-culture, see Methods and Supplementary Information. GFP^+ cells were sorted and cultured on OP9 stromal cells in the presence of IL-7 (left), Epo (middle) and IL-2 (right), as well as SIF and FL. After 7 days in the stromal cell culture, readout cell populations were examined by a flow cytometer. $NK1.1^+$ cells were observed in some cases. The plots shown are results after gating on the $NK1.1^-$ population. **b**, GM colony formation from CLPs by stimulation with GM-CSF. CLPs from wild-type mice were transduced with human GM-CSF receptor by a retroviral vector. Cell culture was done as described in Methods. After 5–8 days in methylcellulose culture, GM colonies were observed. Retrovirally introduced gene expression in three independent GM colonies (lanes 1–3) was confirmed by RT–PCR. **c**, Expression of the indicated genes was determined by RT–PCR in HSCs and CLPs from IL-2Rβ transgenic mice in the presence or absence of human IL-2 (48-h stimulation).

Methods

Mice

Wild-type mice are C57BL/Ka-Thy1.1. Human IL-2Rβ transgenic mice, originally bred on the C57BL/6 background[13], were backcrossed to C57BL/Ka-Thy1.1 over two generations. Mice were bred and maintained in the animal care facility at Stanford University School of Medicine and were used at 5–10 weeks of age.

Flow cytometric analysis and cell sorting

We purified CLPs as described[5]. The surface phenotypes of thymic pro-T and bone marrow pro-B cells used in this study are $CD3^-CD8^-c\text{-}Kit^{high}IL\text{-}2R\alpha^-IL\text{-}7R\alpha^+$(ref. 25) and $IgM^-B220^-CD43^+HSA^-NK1.1^-$(refs 26, 27), respectively. All cell sorting and flow cytometric analysis were done by a highly modified double or triple laser FACS Vantage available at the Stanford shared FACS facility.

Plasmid construction

We cloned cDNAs of human IL-2Rβ and its mutants into the *Eco*RI site of the retroviral vector, MSCV-IRES–GFP (MSCV-2Rβ-IRES–GFP, MSCV-dS-IRES–GFP, MSCV-dA-

IRES–GFP and MSCV-dH-IRES–GFP). The dS, dA and dH mutants lack the S region (amino acid residues 267–322), the A region (residues 313–382) and H region (residues 383–525), respectively. We created MSCV-IL-7Rα-IRES–GFP by inserting a mouse IL-7Rα cDNA into the *Xho*I site of MSCV-IRES–GFP. To generate the MSCV-GMRα-IRES-β_c vector, we inserted human GMRα cDNA into the *Xho*I site of the MSCV-IRES–GFP vector (MSCV-GMRα-IRES–GFP) and put the human β_c cDNA downstream of the IRES in place of the GFP cDNA. A retroviral vector for EpoR (MSCV-EpoR-IRES–GFP)[28] was provided by G. Q. Daley.

Retroviral gene transfer

We produced virus from a packaging cell line, Phoenix, by transient transfection of MSCV constructs with FuGENE 6 (Roche Molecular Biochemicals). For viral infection, CLPs were cultured at 5,000 cells per well in a 96-well round-bottomed plate with 50% virus stock in Iscove's Modified Dulbecco's Media (IMDM) containing 10% FCS, 50 μM 2-mercaptoethanol, 4 μg ml^{-1} polybrene, SIF (20 ng ml^{-1}), FL (20 ng ml^{-1}) and IL-7 (10 ng ml^{-1}) for 12 h at 32 °C. Cells were further cultured for 36–48 h at 37 °C to allow exogenous gene expression. Human IL-2 (25 ng ml^{-1}), erythropoietin (4 U ml^{-1}) and GM-CSF (20 ng ml^{-1}) were used in this culture for stimulating the human IL-2Rβ-, EpoR- and GM-CSFR-expressing CLPs, respectively.

In vitro cell culture

Between 1 and 40 CLPs were sorted by the automatic cell deposition unit (ACDU, Beckton Dickinson) on the FACS Vantage. The cells were cultured in 96-well plates on an OP9 stromal layer in the presence of indicated cytokines for 7–10 days. Methylcellulose culture was done as described[5].

RT–PCR analysis

We isolated RNA from cells with Trizol reagent (GIBCO BRL). Oligo-dT primed cDNA was subjected to PCR. After electrophoresis with 2% agarose gel, bands were detected by ethidium bromide staining. Amplified bands for human GM-CSFRα were detected by Southern blotting with the radiolabelled synthesized oligo as a probe. For the primer sequence, see Supplementary Information.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia

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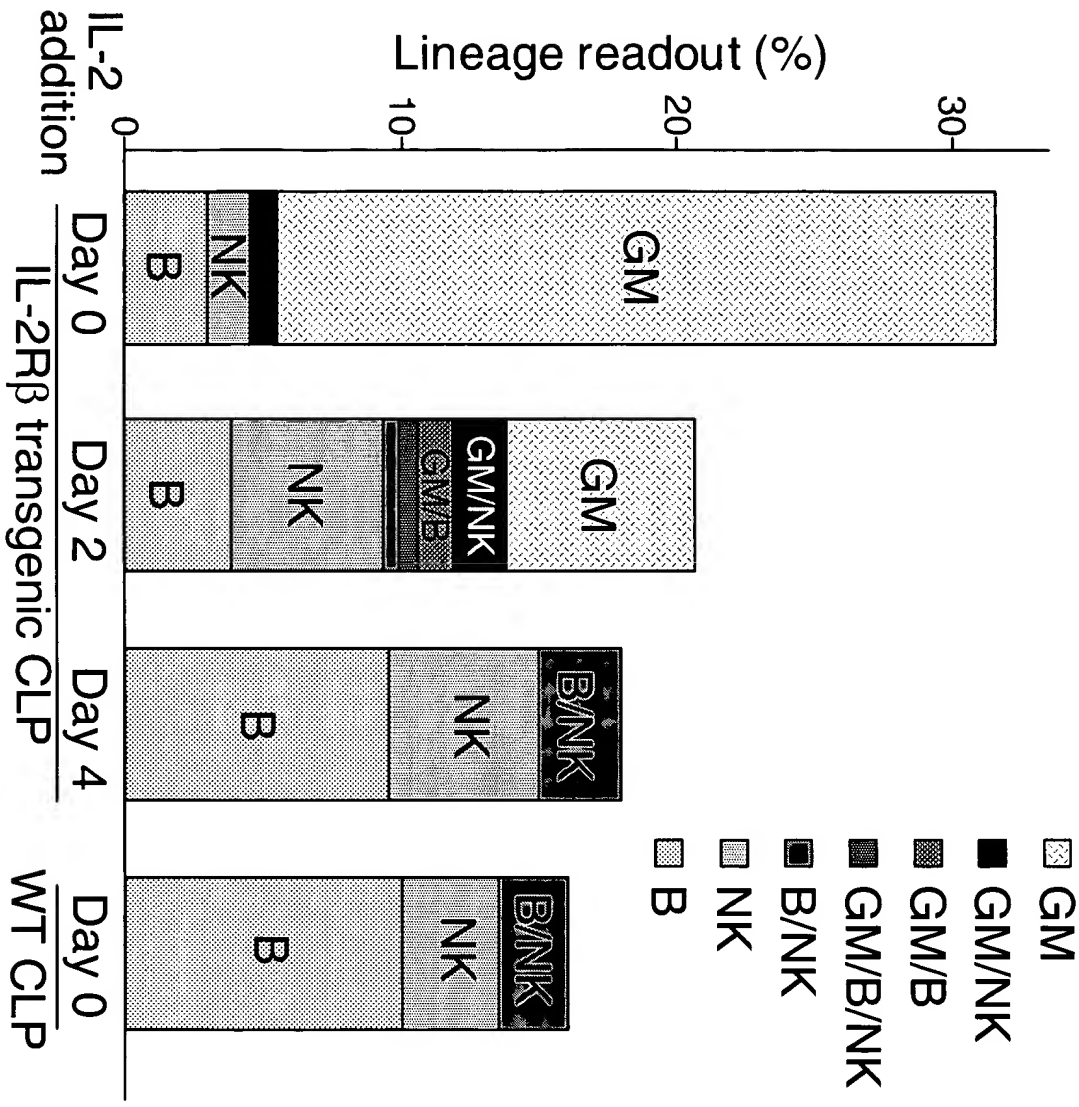
Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections are characterized by early peaks of viraemia that decline as strong cellular immune responses develop[1,2]. Although it has been shown that virus-specific CD8-positive cytotoxic T lymphocytes (CTLs) exert selective pressure during HIV and SIV infection[3–11], the data have been controversial[12,13]. Here we show that Tat-specific CD8-positive T-lymphocyte responses select for new viral escape variants during

EXHIBIT 3


Lineage readout (%)
0
10
20
30
IL-2 addition
Day 0
Day 2
Day 4
Day 0
IL-2Rβ transgenic CLP
WT CLP
B
NK
GM
GM/NK
GM/B
GM/B/NK
B/NK
NK
B